EXHIBIT D

Methods to Increase the Percentage of Free Fetal DNA Recovered From the Maternal Circulation

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Abstract

Context Noninvasive prenatal diagnostic tests using free fetal DNA provide an alternative to invasive tests and their attendant risks; however, free fetal DNA exists in the maternal circulation at low percentages, which has hindered development of noninvasive tests.

Objective To test the hypothesis that using formaldehyde to reduce cell lysis could increase the relative percentage of free fetal DNA in samples of maternal blood.

Design, Setting, and Patients The first phase of the study was conducted from January through February 2002 at a single US clinical site; 2 samples of blood were collected from each of 10 pregnant women, and the percentage of free fetal DNA in formaldehyde-treated and untreated samples was determined. The second phase of the study was conducted from March 2002 through May 2003, and measured the percentage of free fetal DNA in 69 formaldehyde-treated samples of maternal blood obtained from a network of 27 US clinical sites in 16 states.

Main Outcome Measure Percentage of free fetal DNA in samples of maternal blood.

Results In the first phase of the study, the mean percentage of free fetal DNA in the untreated samples was 7.7% (range, 0.32%-40%), while the mean percentage of free fetal DNA in the formaldehyde-treated samples was 20.2% (range, 1.6%-40%) (P = .02 for difference). In the second phase, a median of 25% (range, 3.1% to >50%) free fetal DNA was obtained for the 69 formaldehyde-treated maternal blood samples. Approximately 59% of the samples in this study had 25% or greater fetal DNA, and only 16% of the samples had less than 10% fetal DNA. In addition, 27.5% of the samples in this study had 50% or greater fetal DNA.

Conclusion Addition of formaldehyde to maternal blood samples, coupled with careful processing protocols, increases the relative percentage of free fetal DNA, providing a foundation for development of noninvasive prenatal diagnostic tests to distinguish fetal DNA from maternal DNA in the maternal circulation.

Prenatal diagnosis is useful for managing a pregnancy with an identified fetal abnormality and may allow for planning and coordinating care during delivery and the neonatal period.[1] A variety of prenatal diagnostic tests are available but have limitations. Noninvasive tests such as maternal serum marker testing and ultrasound can be used to screen for the presence of chromosomal abnormalities but are not definitive.[2-5] On the other hand, invasive diagnostic tests (eg, amniocentesis, chorionic villus sampling, percutaneous umbilical blood sampling) for fetal chromosomal abnormalities are highly reliable, but the procedure used for each test carries a risk for loss of pregnancy.[6,7] Many patients who are candidates for these tests decline them because of the risk of pregnancy loss.

An alternative to existing methods for prenatal diagnosis is to use fetal cells and fetal DNA that exist in the maternal circulation.[8-15] Circulating fetal DNA has been used to determine the sex of the fetus through detection of sequences present on the Y chromosome.[13] In addition, several studies have attempted to use free fetal DNA to screen for chromosomal abnormalities in the fetus.[16-21] However, the use of free fetal DNA for detecting chromosomal abnormalities has been limited by the seemingly low percentage of free fetal DNA in the maternal circulation. Lo et al[13] reported a mean of 3.4% free fetal DNA in maternal plasma in the late first to the mid second trimester and a mean of 6.2% free fetal DNA in the late third trimester. Any method that can increase the relative percentage of free fetal DNA in the sample would make it easier to distinguish fetal DNA from maternal DNA. Noninvasive prenatal diagnostic tests that are DNA-based would benefit from higher percentages of free fetal DNA in the samples.

We hypothesized that inhibiting cell lysis during sample collection, shipping, handling, and processing would permit the recovery of a larger percentage of free fetal DNA. By decreasing the amount of maternal cell lysis, and thus the amount of free maternal DNA, the relative percentage of free fetal DNA likely can be increased.

Methods

Blood samples were collected from women carrying a male or a female fetus; however, the majority of samples (81 of 85 [95.3%]) analyzed were obtained from women carrying a male fetus. The Y chromosome is the accepted marker for quantitating percentages of fetal DNA. Each clinical site received institutional review board approval for participation in this research protocol. All women were aged 18 years or older, had a singleton pregnancy, and provided written informed consent prior to enrollment.

Collection of Blood Samples

First Phase. The first phase of this study was conducted from January through February 2002, and recruited women pregnant with a male fetus (identified by ultrasound). Blood samples were collected from the women at a single clinical site prior to an amniocentesis

procedure. Two tubes of blood (9 mL in each tube) were collected from each of 10 women. One tube was treated with 0.225 mL of a 10% neutral buffered solution containing formaldehyde (4% weight per volume) (Sigma, St Louis, Mo), a chemical that stabilizes cell membranes and impedes cell lysis. The other tube was left untreated. The tubes were assigned a numerical code and hand-delivered to our facility for analysis. Laboratory personnel were blinded as to which specimens contained formaldehyde.

Second Phase. For the second phase of the study, conducted from March 2002 through May 2003, a network of 27 clinical sites, operating in 16 states in the United States, was established to collect formaldehyde-treated blood samples from pregnant women prior to amniocentesis or chorionic villus sampling. Seventy-one samples from women carrying a male fetus and 4 samples from women carrying a female fetus were analyzed in this phase of the study. Fetal sex was confirmed by amniocentesis or chorionic villus sampling report. Each sample was coded so that laboratory personnel did not know whether it was obtained from a woman carrying a male or a female fetus.

All samples collected in the second phase of the study were treated with formaldehyde. The clinical sites were provided with a kit used for the venipuncture procedure, which included 21-gauge needles, 9-mL EDTA blood collection tubes, a syringe for each tube containing 0.225 mL of a 10% neutral buffered solution containing formaldehyde (4% weight per volume), an ice pack, and a shipping container. The clinical sites were instructed to add the formaldehyde to the tubes and gently invert them immediately after blood was drawn. The specimens were shipped by commercial carrier for overnight delivery to our facility.

Isolation of Plasma and DNA

The protocols for isolation of plasma were optimized to reduce cell lysis. Tubes were centrifuged at 200*g* for 10 minutes with the brake and acceleration powers set to zero. Tubes then were centrifuged at 1600*g* for 10 minutes with the brake and acceleration powers set to zero. The supernatant (ie, the plasma) of each sample was transferred to a new tube and spun at 1600*g* for 10 minutes with the brake and acceleration powers set to zero. The plasma was transferred carefully to a new tube and stored at −80°C. Approximately 0.5 mL of supernatant was left in the tube to ensure that the buffy coat was not disturbed.

DNA was isolated from plasma samples using the QIAamp DNA Blood Midi Kit (Qiagen, Valencia, Calif) for purification of DNA from blood cells, according to the manufacturer's instructions. DNA was eluted in 100 µL of distilled water.

Primer Design

Two sets of primers were used: 1 set amplified the sex-determining region Y gene (*SRY*), which is located on the Y chromosome and is thus representative of fetal DNA, and the other set amplified the cystic fibrosis gene (*CYS*), which is present on both maternal template DNA and fetal template DNA. Unique regions of the *SRY* gene and the *CYS* gene were identified by sequence searches using the Blast program available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

The following primers were designed to amplify the *SRY* gene: upstream primer: 5' TGGCGATTAAGTCAAATTCGC 3'; downstream primer: 5' CCCCCTAGTACCCTGACAATGTATT 3'. The following primers were designed to amplify the *CYS* gene: upstream primer: 5' CTGTTCTGTGATATTATGTGTGGT 3'; downstream primer: 5' AATTGTTGGCATTCCAGCATTG 3'.

Gene Amplification

The *SRY* gene and the *CYS* gene were amplified from plasma free DNA by polymerase chain reaction (PCR) using the HotStarTaq Master Mix kit (Qiagen). Each PCR reaction used 8 µL of template DNA (diluted or undiluted), 1 µL of each primer (5 µM), and 10 µL of HotStarTaq mix. The following PCR conditions were used: (1) 95°C for 15 minutes, (2) 94°C for 30 seconds, (3) 54°C for 15 seconds, (4) 72°C for 30 seconds, (5) repeat steps 2 through 4 for 45 cycles, and (6) 72°C for 10 minutes.

Quantification of Fetal DNA

The percentage of free fetal DNA in the maternal plasma sample was determined by PCR using serially diluted plasma DNA, which accurately quantifies the number of genomes that harbor the amplified gene. For example, if the blood sample contains 100% male fetal DNA, and 1:2 serial dilutions are performed, then on average the *SRY* signal will disappear 1 dilution before the *CYS* signal, since there is 1 copy of the *SRY* gene and 2 copies of the *CYS* gene.

The percentage of free fetal DNA in the maternal plasma was calculated using the following formula: percentage of free fetal DNA = (No. of copies of *SRY* gene × 2 × 100)/(No. of copies of *CYS* gene), where the number of copies of each gene was determined by observing the highest serial dilution in which the gene was detected. The formula contains a multiplication factor of 2, which is used to normalize for the fact that there is only 1 copy of the *SRY* gene.

In the first phase of the study, 6 serial dilutions (1:5) were performed for each sample (1:5 to 1:15625). Provided that there is at least 1 copy of the *SRY* gene and that the *CYS* gene is detected in the sixth serial dilution, the lowest possible value is 0.0128% free fetal DNA ([1 × 2 × 100]/15625). All other values increase by multiples of 5 from 0.0128, depending on the number of dilutions that were positive for the *SRY* and *CYS* genes (eg, 0.064, 0.32, 1.6, 8, and 40).

In the second phase of the study, 10 serial dilutions (1:2) were performed for each sample (1:2 to 1:1024). Provided that there is at least 1 copy of the *SRY* gene and that the *CYS* gene is detected in the 10th serial dilution, the lowest possible value is 0.1953% free fetal DNA ([1 × 2 × 100]/1024). All other values increase by multiples of 2 from 0.1953.

The number of fetal genomes per milliliter of plasma was calculated using the following formula: No. of genomes/mL of plasma = (No. of copies of *SRY* gene/volume of DNA in reaction [μL]) × (volume of DNA eluted [μL]/total volume of plasma through column [mL]).

Statistical Analysis

The primary outcome in the first phase of the study was the difference in the percentage of free fetal DNA between formaldehyde-treated and untreated samples. The nonparametric Wilcoxon signed rank test, which assumes that there is information in the magnitude of differences, was used to analyze the data from the first phase of the study. All analyses were performed using Analyse-it General & Clinical Laboratory Statistics, version 1.71 (Analyse-it Software Ltd, Leeds, England); $P<.05$ was used to determine statistical significance.

Results

First Phase

The results from the first phase of the study are summarized in Table 1. Analysis of the untreated samples revealed a mean of 7.7% (range, 0.32%-40%) free fetal DNA. The formaldehyde-treated samples had a mean of 20.2% (range, 1.6%-40%) free fetal DNA.

Several of the untreated samples (from participants 1, 3, 4, and 10) contained percentages of free fetal DNA that were substantially below the median. Even with these samples, the addition of formaldehyde increased the relative percentage of free fetal DNA. For instance, in untreated samples from participants 4 and 10, the percentage of free fetal DNA was 0.32%, whereas in foraldehyde-treated samples collected from the same women the percentage of free fetal DNA was increased to 8%.

In 3 of the samples (from participants 2, 6, and 7), there was no measurable effect of formaldehyde on the percentage of free fetal DNA. However, analysis of the paired samples from the first phase of the study using the Wilcoxon signed rank test revealed that, overall, the addition of formaldehyde significantly increased the percentage of free fetal DNA (P = .02; W = 28 for y = 7).

Second Phase

Because analysis of the samples from the first phase of the study revealed that the effect of formaldehyde was statistically significant, the second phase of the study was designed to evaluate the percentage of free fetal DNA in formaldehyde-treated samples in a larger patient population from multiple clinical sites. A total of 75 samples from pregnant women were collected in this phase of the study. Seventy-one samples were collected from women who carried a male fetus. However, 2 samples were excluded from analysis because these samples were not received within 24 hours after collection.

Four samples were obtained from women who carried a female fetus. For each of these samples, a robust signal was observed for the *CYS* gene; as expected, no signal was detected for the *SRY* gene, which is specific for the Y chromosome.

Analysis of the 69 formaldehyde-treated samples revealed a median of 25% (range, 3.1% to >50%) free fetal DNA (Table 2). Approximately 16.0% of the samples (11/69) had less than 10% free fetal DNA; approximately 59% of the formaldehyde-treated samples had 25% or greater free fetal DNA and 27.5% of the samples had 50% or greater free fetal DNA (4 [5.8% of total] samples had 3.1% free fetal DNA; 7 [10.1%] had 6.2%; 17 [24.6%] had 12.5; 22 [31.9%] had 25%; 8 [11.6%] had 50%; and 11 [15.9%] had >50%). Although many of the formaldehyde-treated samples contained high percentages of free fetal DNA, there was variability in the percentages obtained.

Analysis of the formaldehyde-treated samples also revealed a mean of 66.1 fetal genomes/mL of plasma, with a range of 3.0 fetal genomes/mL to 533 fetal genomes/mL (Table 2). Some of the samples (eg, sample 12) had a limited number of fetal genomes but a high percentage of fetal DNA. Conversely, some samples had an ample number of fetal genomes but a lower percentage of fetal DNA. For instance, sample 40 had 112.5 fetal genomes/mL but the percentage of fetal DNA was 6.2% (Table 2).

Comment

We have shown that the relative percentage of free fetal DNA recovered from maternal blood samples can be increased. Addition of formaldehyde to maternal blood samples, coupled with careful processing protocols, resulted in an increase in the percentage of free fetal DNA recovered from the maternal circulation. This increase in the relative percentage of free fetal DNA likely resulted from a combination of factors.

First, formaldehyde stabilizes cell membranes, thereby preventing cell lysis and the release of DNA. Prior to the venipuncture procedure, the amount of free maternal DNA in the maternal circulation likely is low. However, the maternal cells may lyse during sample collection, shipping, handling, and processing. For example, during centrifugation the cells are exposed to gravitational forces, which may rupture cells. The presence of formaldehyde protects the cells from lysis.

Second, the addition of formaldehyde may allow a larger recovery of free fetal DNA by inhibiting enzymes that destroy DNA, such as DNases. For the samples analyzed in the second phase of the study, a mean of 66.1 fetal genomes/mL was obtained, which represents a 2.6-fold increase over the mean reported in the literature (25.4 fetal genomes/mL).[13] Inhibition of enzymes that destroy DNA would permit a larger recovery of DNA (including free fetal DNA) already present in the sample. Also, the addition of formaldehyde may stabilize and preserve the structure of DNA, which may increase the amount of DNA recovered.

Third, in conjunction with the addition of formaldehyde to the maternal blood samples, sample-processing protocols designed to minimize cell lysis likely contributed to increases in the percentage of free fetal DNA. A centrifugation protocol was designed to minimize gravitational forces imposed on the cells. Samples initially were spun at a low speed, which allowed the majority of cells to separate from the plasma under minimal forces. In addition, all centrifugation steps were performed with the acceleration and brake powers set to zero. This reduced the formation of a vortex during centrifugation, minimizing mixing of the plasma and the buffy coat, which contains maternal cellular material. Also, when removing the plasma sample, care was taken to ensure that the buffy coat was not disturbed.

A larger study would be useful to delineate factors contributing to the results presented in this study, and to understand why some samples have a higher percentage of free fetal DNA than others. For instance, in 3 samples from the first phase of the study, there was no measurable effect of formaldehyde on the percentage of free fetal DNA. It is possible that formaldehyde was not added or mixed properly with the treated samples or may have been added to both treated and untreated samples.

Also, in some samples there may be a greater amount of free maternal DNA already present in the maternal circulation. While the addition of formaldehyde will impede cell lysis that occurs during sample collection, shipping, handling, and processing, it likely will not reduce the concentration of free maternal DNA already present in the sample. A larger study comparing the percentage of free fetal DNA in formaldehyde-treated and untreated samples will help to address these issues.

Furthermore, several controllable factors likely contribute to the variation in the percentages of free fetal DNA. One such factor is the time interval between the venipuncture procedure and sample processing. The shorter this time interval the more likely it is the integrity of the sample will be preserved and cell lysis kept to a minimum. Similarly, the length of time between the venipuncture procedure and the addition of formaldehyde is thought to be critical. If formaldehyde is not added shortly after the venipuncture procedure, cells may lyse and release DNA. Also, it is important that the formaldehyde is gently mixed throughout the tube to allow maximum exposure to the reagent.

With an increased percentage of free fetal DNA in the maternal blood samples, the sequence of fetal DNA can be discerned from maternal DNA using natural genetic markers, such as single nucleotide polymorphisms. For example, at certain genomic sites, the maternal genome will be homozygous for allele A, while the paternal genome is homozygous for allele B, which means the fetal genome will be heterozygous at this genomic site. Allele B represents a distinct fetal signal in the maternal blood sample. The detection and quantitation of fetal DNA, in this case allele B, is more attainable with an increased percentage of fetal DNA, and can be used to diagnose single-gene disorders and chromosomal abnormalities.

A ratio for alleles A and B can be quantitated and used to detect chromosomal disorders. When samples have a high percentage of free fetal DNA, the difference between the expected ratio of the chromosomes for a healthy fetus and that for an abnormal fetus is greater, which makes it easier to diagnose chromosomal abnormalities. Thus, the methods described herein for increasing the percentage of free fetal DNA provide a solid foundation for the development of a noninvasive prenatal diagnostic test.

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